

# Sox2 functions as a sequence-specific DNA sensor in neutrophils to initiate innate immunity against microbial infection

Pengyan Xia<sup>1,2</sup>, Shuo Wang<sup>1,2</sup>, Buqing Ye<sup>1</sup>, Ying Du<sup>1</sup>, Guanling Huang<sup>1</sup>, Pingping Zhu<sup>1</sup> & Zusen Fan<sup>1</sup>

Neutrophils express Toll-like receptors (TLRs) for the recognition of conserved bacterial elements to initiate antimicrobial responses. However, whether other cytosolic DNA sensors are expressed by neutrophils remains elusive. Here we found constitutive expression of the transcription factor Sox2 in the cytoplasm of mouse and human neutrophils. Neutrophil-specific Sox2 deficiency exacerbated bacterial infection. Sox2 directly recognized microbial DNA through its high-mobility-group (HMG) domain. Upon challenge with bacterial DNA, Sox2 dimerization was needed to activate a complex of the kinase TAK1 and its binding partner TAB2, which led to activation of the transcription factors NF- $\kappa$ B and AP-1 in neutrophils. Deficiency in TAK1 or TAB2 impaired Sox2-mediated antibacterial immunity. Overall, we reveal a previously unrecognized role for Sox2 as a cytosolic sequence-specific DNA sensor in neutrophils, which might provide potential therapeutic strategies for the treatment of infectious diseases.

Neutrophils, a major component of the innate immune response, represent the largest population among circulating white blood cells in humans. Congenital defects in neutrophils lead to fatal infections<sup>1,2</sup>. Neutrophils reach the site of inflammation caused by bacterial infection at a very early stage, acting as the first defense line against invading pathogens<sup>3,4</sup>. Upon recruitment to the site of inflammation, neutrophils use three main means to kill bacteria: the engulfment of microbes through receptor-mediated phagocytosis, the release of granular antimicrobial factors via degranulation, and the generation of neutrophil extracellular traps<sup>1,5</sup>. In addition to directly killing invasive pathogens, neutrophils also potentiate the host immune response by producing cytokines<sup>3,6</sup>. The sensing of microbes by pattern-recognition receptors (PRRs) expressed by neutrophils results in the production of proinflammatory cytokines and chemokines, which are necessary for the recruitment of other cells of the immune system to the inflammatory area for the elimination of bacteria. Neutrophils can express Toll-like receptors (TLRs) for the recognition of conserved bacterial elements<sup>7</sup>, collectively called 'pathogen-associated molecular patterns'. These members of the TLR family reside on the lumen of endosomal membranes in neutrophils to monitor various forms of nucleic acids of foreign microbes. However, whether other cytosolic sensors of nucleic acids in neutrophils can discriminate bacterial genomic DNA from host DNA remains elusive.

Nucleic acid sensors can recognize the DNA or RNA of invading microbes to trigger host antimicrobial responses such as the generation of type I interferons and proinflammatory cytokines<sup>8–10</sup>. Several cytosolic DNA sensors that monitor cytosolic microbial DNA have

been identified so far. TLR9 in plasmacytoid dendritic cells can sense exogenous CpG DNA to activate signaling via the transcription factors NF- $\kappa$ B and IRF7 (ref. 11). The cytosolic receptor AIM2 associates with AT-rich double-stranded DNA (dsDNA) to activate NF- $\kappa$ B and caspase-1 upon inflammasome formation<sup>12</sup>. The cytosolic receptor DAI (also known as DLM1 or ZBP1) can recognize exogenous AT-rich DNA in some cell lines<sup>13,14</sup>. The DNA sensor IFI16, originally identified in THP-1 human monocyte cells, recognizes intracellular B-form DNA to initiate the production of type I interferons<sup>15</sup>. The helicase DDX41 has been reported to bind cytosolic DNA for the induction of type I interferons in dendritic cells<sup>16</sup>. However, the DNA-sensing role of IFI16 and DDX41 has not been confirmed by genetic loss-of-function studies so far. The enzyme cyclic GMP-AMP synthase (cGAS) has been reported to bind cytoplasmic DNA as a DNA sensor<sup>17–19</sup>, which initiates the production of type I interferons and other inflammatory cytokines. This DNA binding activates cGAS to synthesize the specific cGAMP isomer 2'3'-cGAMP, which causes activation of the adaptor STING and then leads to innate immune responses.

Sox2 is a transcription factor known for its essential role in maintaining the self-renewal of embryonic stem (ES) cells and the formation of ectodermal and endodermal tissues during fetal development<sup>20,21</sup>. Sox2 contains a high-mobility-group (HMG) domain, a group B homolog (GBH) domain, a serine-rich domain (SRD) and two transcription-activating domains<sup>20,22</sup>. Sox2 belongs to the Sox family of transcription factors and is essential for cell-fate determination<sup>23,24</sup>. Sox2 is expressed in the inner cell mass of

<sup>1</sup>Key Laboratory of Infection and Immunity of CAS, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. <sup>2</sup>These authors contributed equally to this work. Correspondence should be addressed to Z.F. (fanz@moon.ibp.ac.cn).

Received 23 November 2014; accepted 28 January 2015; published online 2 March 2015; doi:10.1038/ni.3117

the blastocyst, and Sox2 deficiency impairs the formation of the pluripotent epiblast, which leads to death *in utero*<sup>25</sup>. Together with the transcription factors c-Myc, KLF4 and Oct-4, Sox2 can reprogram differentiated somatic cells into induced pluripotent stem cells<sup>26,27</sup>. Sox2 binds in the promoter of the gene encoding the metabolic checkpoint kinase mTOR to inhibit its transcription at the early stage of reprogramming mouse embryonic fibroblasts into induced pluripotent stem cells<sup>28</sup>. Here we found that Sox2 was constitutively expressed in the cytoplasm of neutrophils and, through its HMG domain, as a DNA sensor, recognized bacterial DNA. Dimerization of Sox2 was required for activation of a complex of the kinase TAK1 and its binding partner TAB2 that was essential for the induction of proinflammatory cytokines in neutrophils.

## RESULTS

### Sox2 expression in neutrophil cytoplasm

While investigating the expression pattern of Sox2 in hematopoietic stem cells (HSCs) in mouse bone marrow (BM) through immunohistochemical staining, we observed Sox2<sup>+</sup> cells whose morphology resembled that of neutrophils. Flow cytometry of mouse peripheral blood cells showed that most CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils also stained with antibody to Sox2 (anti-Sox2) (Fig. 1a). The CD11b<sup>+</sup>Ly6G<sup>+</sup> Sox2<sup>+</sup> neutrophils also expressed myeloperoxidase (MPO), an enzyme present in the granules of neutrophils, which serves as a neutrophil marker<sup>1</sup> (Fig. 1a). Additionally, we detected the expression of Sox2 mRNA and Sox2 protein in isolated peripheral neutrophils, through RT-PCR and immunoblot analysis, respectively (Fig. 1a). However, Sox2 was not expressed in other hematopoietic cells we tested, such as T cells, B cells, natural killer cells, dendritic cells and macrophages (Fig. 1b), or other granulocytes, such as basophils and eosinophils (Fig. 1c). Sox2 was expressed in mouse embryonic stem (ES) cells, which served as a positive control (Fig. 1b). These data indicated that among mature hematopoietic cells, only neutrophils expressed Sox2.

To further determine the subcellular localization of Sox2, we resolved cytosolic and nuclear neutrophil extracts via subcellular fractionation. In neutrophils, Sox2 was localized to the cytoplasm (Fig. 1d). We confirmed that observation by immunostaining of

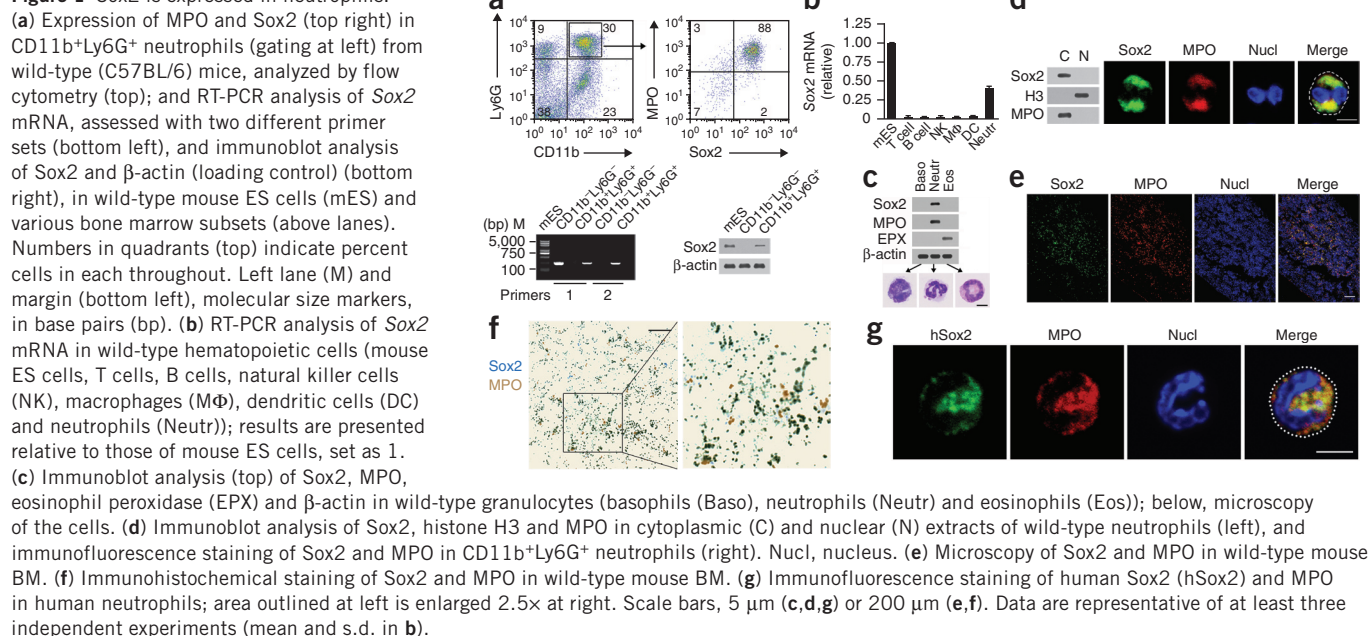
neutrophils with anti-Sox2 and anti-MPO (Fig. 1d). We tested several different antibodies to Sox2 in the immunoblot analysis and fluorescence staining assays and obtained similar results (data not shown). By *in situ* immunostaining of mouse BM, we observed that most MPO<sup>+</sup> cells also stained with anti-Sox2 (Fig. 1e). We verified the costaining of MPO and Sox2 in BM neutrophils by immunohistochemical staining (Fig. 1f). We made similar observations with mouse spleen (Supplementary Fig. 1a,b). Staining of mouse and human ES cells with the same antibodies to Sox2 used in the assays above detected Sox2 expression in the nuclei of ES cells (Supplementary Fig. 1c), which confirmed their specificity for the protein. We also detected Sox2 in the cytoplasm of human peripheral neutrophils (Fig. 1g). Together these observations suggested constitutive expression of Sox2 in the cytoplasm of mouse and human neutrophils.

### Neutrophil-specific Sox2 deficiency aggravates bacterial infection

To explore the physiological role of Sox2 in neutrophils, we crossed mice with loxP-flanked Sox2 alleles (*Sox2*<sup>fl/fl</sup>) with mice expressing Cre recombinase from the myeloid-specific gene *Lyz2* (*Lyz2*-Cre) to generate the mice with conditional deletion of Sox2 in neutrophils (*Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>+</sup> mice) (Supplementary Fig. 2a). Sox2 was completely deleted from *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>+</sup> neutrophils, whereas MPO and  $\beta$ -actin were unchanged and served as loading controls (Fig. 2a). Because the *Lyz2*-Cre system also deletes genes in macrophages, we confirmed that Sox2 was not expressed in *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>-</sup> macrophages *in vivo* (Supplementary Fig. 2b). The number of neutrophils in the BM and peripheral blood of *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>+</sup> mice was similar to that in their *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>-</sup> littermates, which served as controls (Fig. 2b,c). Additionally, the morphology of neutrophils from *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>+</sup> mice was similar to that of neutrophils from their *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>-</sup> littermates (Fig. 2d). These data suggested that neutrophil-specific Sox2 deficiency did not affect the development of neutrophils.

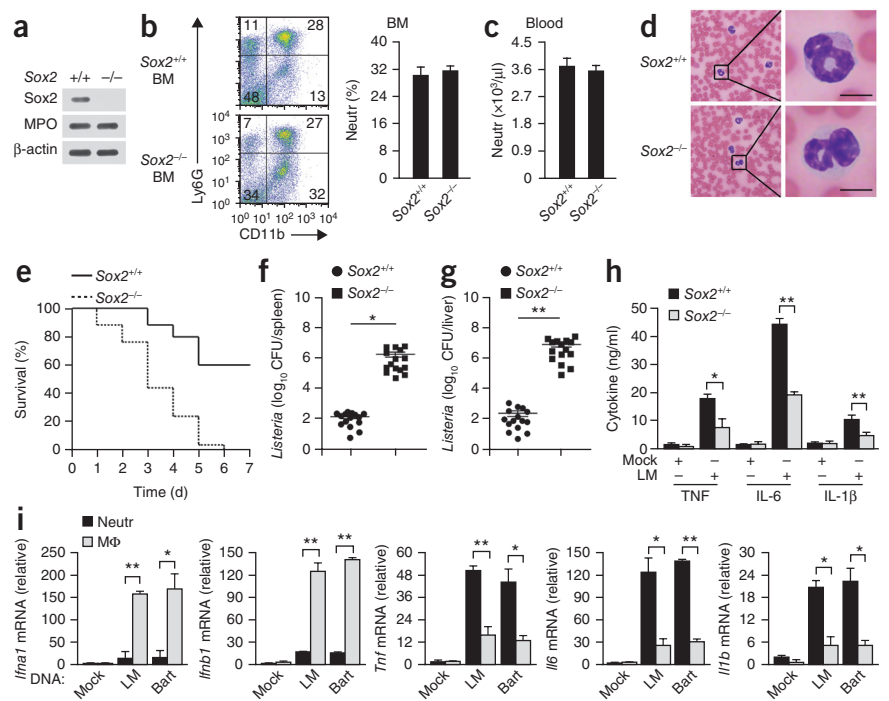
To determine whether Sox2 has a role in neutrophils during the clearance of bacteria *in vivo*, we infected *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>+</sup> mice and their *Sox2*<sup>fl/fl</sup>*Lyz2*-Cre<sup>-</sup> littermates with  $2 \times 10^6$  *Listeria monocytogenes*, which is detected by DNA sensors in other types of myeloid cells.

**Figure 1** Sox2 is expressed in neutrophils.



**Figure 2** Sox2 deficiency in neutrophils aggravates bacterial infection.

(a) Immunoblot analysis of Sox2, MPO and  $\beta$ -actin in CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils from Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> (Sox2<sup>+/+</sup>) and Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> (Sox2<sup>-/-</sup>) mice. (b,c) Flow cytometry of neutrophils in BM (b) and peripheral blood (c) from mice as in a. (d) Giemsa staining of peripheral neutrophils from mice as in a; area outlined at left is enlarged 6.5 $\times$  at right. Scale bars, 5  $\mu$ m. (e) Survival of mice as in a ( $n = 20$  per group) after intraperitoneal injection of  $2 \times 10^6$  *L. monocytogenes*. (f,g) Plaque assay of homogenized spleen (f) and liver (g) from mice as in a ( $n = 15$  per group) 3 d after intraperitoneal injection of  $2 \times 10^5$  *L. monocytogenes*. CFU, colony-forming units. (h) Enzyme-linked immunosorbent assay (ELISA) of TNF, IL-6 and IL-1 $\beta$  in serum from mice ( $n = 15$  per group) mock infected (Mock +) or infected with *L. monocytogenes* (LM +) as in f,g. (i) RT-PCR analysis of mRNA encoding interferons and proinflammatory cytokines in neutrophils and macrophages mock transfected (Mock) or transfected with DNA from *L. monocytogenes* (LM) or *B. henselae* (Bart); results are presented relative to those of mock-transfected cells, set as 1. Each symbol (f,g) represents an individual mouse; small horizontal lines indicate the mean ( $\pm$  s.d.). \* $P < 0.01$  and \*\* $P < 0.001$  (Student's *t*-test). Data are representative of three experiments with similar results (mean and s.d. in b,c,h,i).

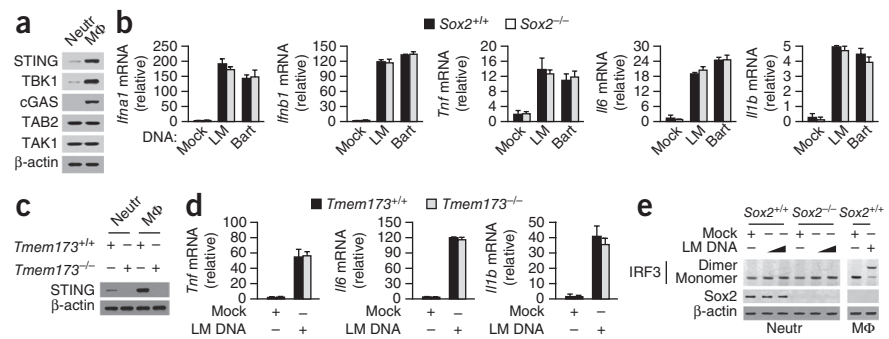


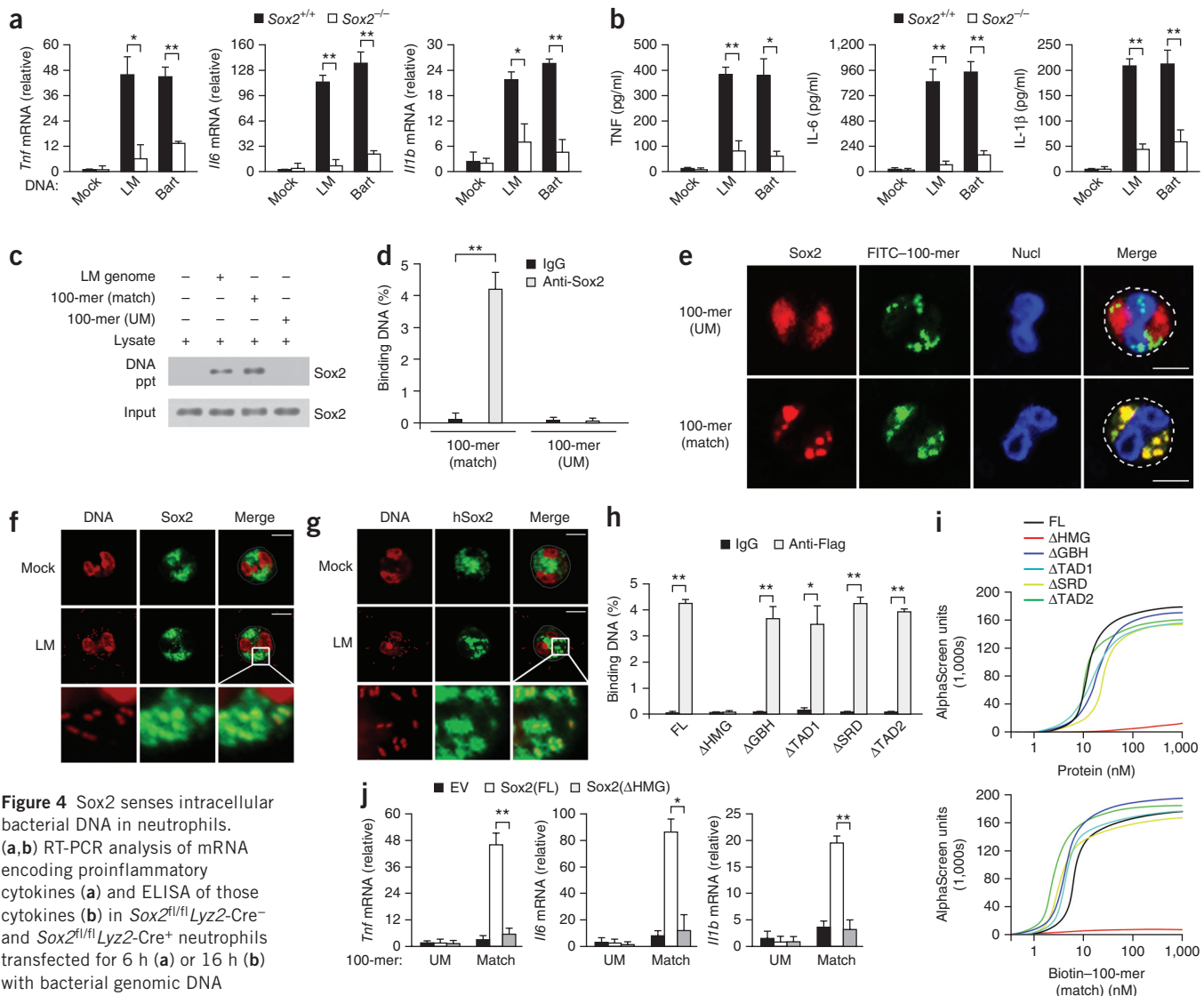
More *L. monocytogenes*-infected Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> mice died than their Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> littermate counterparts (Fig. 2e). Consistent with that, the bacterial load in the spleen and liver was 10,000-fold higher in Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> mice than in their Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> littermates (Fig. 2f,g). Moreover, the concentration of proinflammatory cytokines such as tumor-necrosis factor (TNF), interleukin (IL-6) and IL-1 $\beta$  was two times lower in the serum of Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> mice than in that of their Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> littermates (Fig. 2h). Various DNA sensors have been reported to initiate the generation of type I interferons in most cells of the innate immune system<sup>17</sup>. However, transfection of bacterial genomic DNA triggered the expression of only proinflammatory cytokines, not that of type I interferons, in neutrophils (Fig. 2i).

Because cGAS induces the production of type I interferons and other inflammatory cytokines via STING<sup>17–19</sup>, an adaptor for the cytoplasmic sensing of DNA, we investigated the requirement for cGAS and/or STING in the cytosolic recognition of DNA in neutrophils.

Neutrophils had low expression of STING and undetectable expression of cGAS (Fig. 3a). In contrast, macrophages had high expression of STING and cGAS (Fig. 3a). We found that after bacterial challenge, Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> macrophages and Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> macrophages expressed similar levels of mRNA encoding type I interferons and cytokines such as TNF, IL-6 and IL-1 $\beta$  (Fig. 3b), which suggested that Sox2-deficient macrophages responded normally to cytosolic DNA challenge. To further exclude the possibility of involvement of the cGAS-STING axis in DNA recognition in neutrophils, we used the genome-editing approach based on clustered regularly interspaced short palindromic repeats and the endonuclease Cas9 to achieve complete deletion of STING in neutrophils (Fig. 3c and Supplementary Fig. 2c,d). Following transfection of *L. monocytogenes* genomic DNA, STING-deficient neutrophils and wild-type neutrophils expressed similar levels of mRNA encoding TNF, IL-6 and IL-1 $\beta$  (Fig. 3d). Finally, after transfection of *L. monocytogenes* genomic DNA, IRF3 was not activated

**Figure 3** cGAS or STING is not required for the cytoplasmic recognition of DNA in neutrophils. (a) Immunoblot analysis of innate immunoregulators (left margin) in wild-type neutrophils and macrophages. (b) RT-PCR analysis of mRNA encoding interferons and proinflammatory cytokines in Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> and Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> macrophages transfected with bacterial DNA (as in Fig. 2i). (c) Immunoblot analysis of STING in neutrophils and macrophages from wild-type (*Tmem173*<sup>+/+</sup>) and STING-deficient (*Tmem173*<sup>-/-</sup>) mice. (d) RT-PCR analysis of mRNA encoding proinflammatory cytokines in neutrophils obtained from mice as in c and mock transfected or transfected with *L. monocytogenes* DNA. (e) Immunoblot analysis of IRF3 and Sox2 in Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>-</sup> and Sox2<sup>fl/fl</sup>Ly2z-Cre<sup>+</sup> macrophages mock transfected or transfected with increasing amounts (wedge) of *L. monocytogenes* DNA. mRNA results (b,d) are presented relative to those of mock-transfected cells (the cells in Fig. 2i, for b), set as 1. Data are representative of three experiments with similar results (mean and s.d. in b,d).





**Figure 4** Sox2 senses intracellular bacterial DNA in neutrophils.

(a,b) RT-PCR analysis of mRNA encoding proinflammatory cytokines (a) and ELISA of those cytokines (b) in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>-</sup>* and *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils transfected for 6 h (a) or 16 h (b) with bacterial genomic DNA (as in Fig. 2i). (c) Immunoblot analysis of a DNA-precipitation (ppt) assay of wild-type neutrophil lysates incubated with *L. monocytogenes* genomic DNA (LM genome), the 100-nucleotide matched (100-mer match) or unmatched (100-mer UM) *L. monocytogenes* sequence, probed with anti-Sox2; below (Input), lysates analyzed without precipitation. (d) Chromatin immunoprecipitation, with immunoglobulin G (IgG) or anti-Sox2 (key), from neutrophils transfected with matched or unmatched DNA (as in c), followed by PCR; results are presented as precipitated DNA/total transfected DNA (Binding DNA (%)). (e) Confocal microscopy of Sox2 in wild-type neutrophils transfected for 6 h with fluorescein isothiocyanate (FITC)-conjugated 100-nucleotide matched or unmatched DNA. Dashed outline (far right) indicates neutrophil profile. Scale bar, 5  $\mu$ m. (f,g) Confocal microscopy of Sox2 in mouse (f) and human (g) neutrophils incubated for 2 h with medium alone (Mock) or live *L. monocytogenes*. Area outlined (middle right) is enlarged 4.5 $\times$  in bottom row. Scale bars, 5  $\mu$ m. (h) Chromatin immunoprecipitation, with immunoglobulin G (IgG) or anti-Flag (key), from neutrophils transfected with 100-nucleotide matched *L. monocytogenes* DNA and Flag-tagged full-length (FL) Sox2 or Sox2 mutants lacking ( $\Delta$ ) various domains (horizontal axis), presented as in d. (i) Luminescent proximity assay of 100-nucleotide matched DNA (40 nM) incubated with various concentrations (horizontal axis) of full-length or mutant Sox2 (key) (top) or full-length or mutant Sox2 (40 nM) mixed with various concentrations (horizontal axis) of biotin-labeled 100-nucleotide matched DNA (bottom). (j) RT-PCR analysis of mRNA encoding proinflammatory cytokines in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils transfected with 100-nucleotide matched or unmatched DNA (horizontal axis) and with empty vector (EV) or with vector to express full-length Sox2 (Sox2(FL)) or Sox2 lacking the HMG domain (Sox2( $\Delta$ HMG)) (key). mRNA results (a,j) are presented relative to those of mock-transfected cells (a) or cells transfected with empty vector (j), set as 1. \* $P < 0.01$  and \*\* $P < 0.001$  (Student's *t*-test). Data are representative of three experiments with similar results (mean and s.d. in a,b,d,h,j).

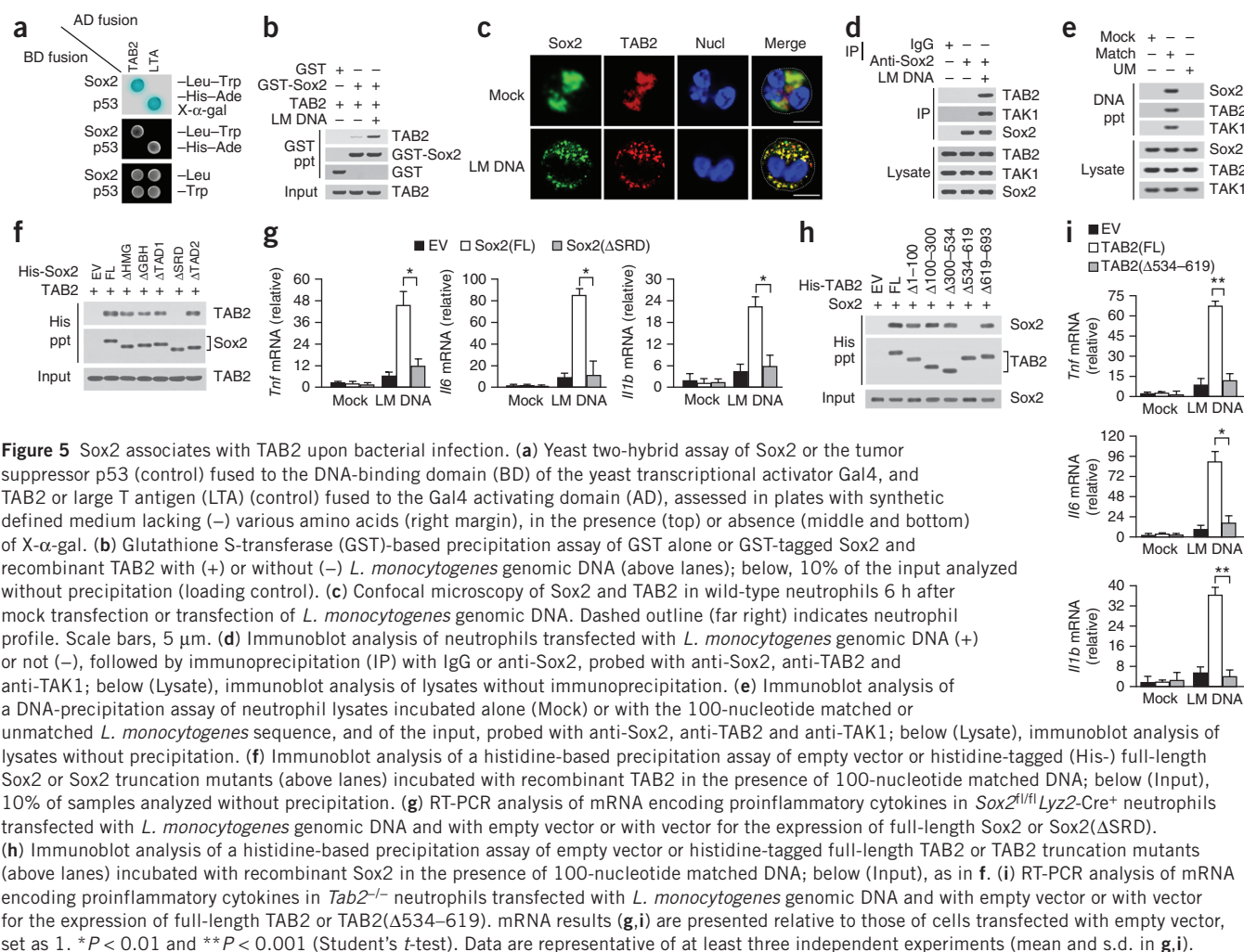
in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>-</sup>* neutrophils, while it was activated in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* macrophages (Fig. 3e). Thus, the Sox2-mediated activation of neutrophils was independent of STING or cGAS.

Notably, we observed that neutrophils from *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* mice and their *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>-</sup>* littermates expressed similar levels of mRNA encoding TNF, IL-6 and IL-1 $\beta$  following stimulation of TLR2 with the synthetic lipopeptide Pam<sub>3</sub>CSK<sub>4</sub>, stimulation of TLR8 with single-stranded RNA or stimulation of TLR4 with lipopolysaccharide

(Supplementary Fig. 2e–g). This suggested that Sox2-deficient neutrophils responded normally to TLR ligands. Collectively, these data suggested a critical role for Sox2 in neutrophil-mediated bacterial clearance that was independent of STING or cGAS.

#### Sox2 recognizes intracellular bacterial DNA in neutrophils

Sox2 contains a DNA-binding motif and binds to well-defined DNA sequences<sup>29</sup>. To test the hypothesis that cytosolic Sox2 in neutrophils



might sense bacterial DNA, we isolated neutrophils from *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* and *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* mice and transfected the cells with bacterial genomic DNA *in vitro*. Following transfection with genomic DNA from *L. monocytogenes* or *Bartonella henselae*, *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* neutrophils produced four- to tenfold less TNF, IL-6 and IL-1 $\beta$  (quantified as mRNA and protein) than did *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* neutrophils (Fig. 4a,b). These results suggested that Sox2 was required for neutrophil activation following challenge with bacterial genomic DNA.

Next we looked for Sox2-binding sequences in the *L. monocytogenes* genome with the alignment search tool BLAST. A large array of regions matched the canonical Sox2-binding sequence (Supplementary Fig. 3a). We selected 30 *L. monocytogenes* DNA sequences that matched the Sox2-binding sequence (called 'matched' here) and one random sequence (called 'unmatched' here) for further verification (Supplementary Fig. 3b). Over 90% of the matched sequences were able to bind to Sox2 (Supplementary Fig. 3c). To investigate whether bacterial DNA can bind to Sox2 directly, we chose 100-nucleotide dsDNA regions of the *L. monocytogenes* genome containing the matched or unmatched sequences (Supplementary Fig. 3d). *In vitro*, the matched dsDNA of the *L. monocytogenes* genome precipitated Sox2 from neutrophil lysates, whereas the unmatched dsDNA of the *L. monocytogenes* genome did not (Fig. 4c). Whole *L. monocytogenes* genomic DNA was similarly able to precipitate Sox2 from neutrophil lysates (Fig. 4c). Following transfection of the 100-nucleotide matched and unmatched dsDNA into neutrophils,

the matched dsDNA associated with Sox2 in neutrophils and induced the production of TNF, IL-6 and IL-1 $\beta$ , while the unmatched dsDNA did not associate with Sox2 (Fig. 4d and Supplementary Fig. 3e). Notably, Sox2 localized together with bacterial DNA in mouse neutrophils following transfection of matched dsDNA or infection with *L. monocytogenes* (Fig. 4e,f). Similarly, we observed colocalization of Sox2 and bacterial DNA in human neutrophils upon infection with *L. monocytogenes* (Fig. 4g).

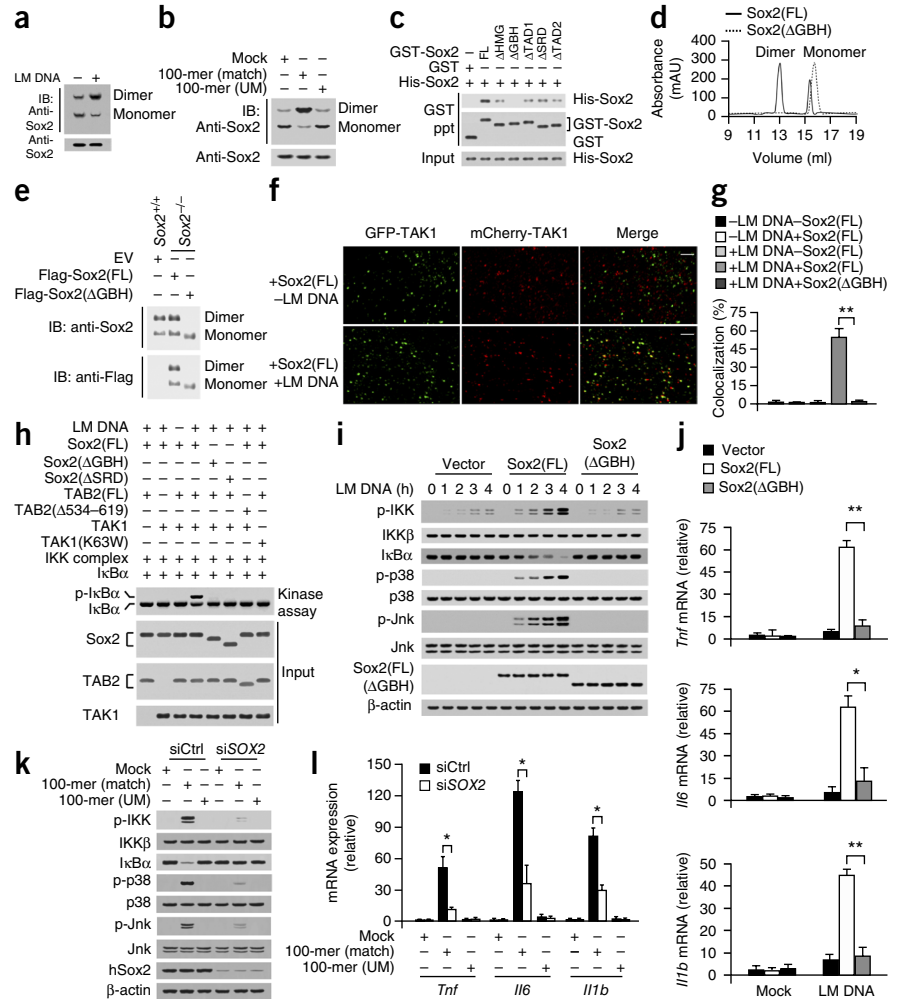
We also searched other bacterial genomes, such as those of *Bartonella*, *Staphylococcus* and *Salmonella* species, using the canonical Sox2-binding sequence. We found that many DNA fragments that matched the Sox2-binding sequence existed in these bacterial genomes and bound to Sox2 (Supplementary Fig. 3f–h). In contrast, unmatched DNA fragments used as negative controls were unable to bind Sox2 (Supplementary Fig. 3f–h). When transfected into neutrophils, these matched DNA sequences induced the expression of proinflammatory cytokines (Supplementary Fig. 3i–k), which suggested that Sox2 recognizes bacterial DNA in a sequence-specific manner. We also tested the effect of neutrophil-specific deletion of Sox2 on infection with vaccinia virus. Viral loads were similar in *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* and *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* mice following infection with this virus (Supplementary Fig. 4a,b). We detected similar levels of type I interferon expression in vaccinia virus-infected *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* and *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* macrophages, while *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* and *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* neutrophils expressed very little interferon

**Figure 6** The Sox2 dimer is required for activation of the TAB2-TAK1 complex.

(a) Immunoblot analysis (IB) of Sox2 in neutrophils left unchallenged (–) or challenged with *L. monocytogenes* genomic DNA (+), assessed for proteins separated by native PAGE (top) or SDS-PAGE (bottom).

(b) Immunoblot analysis of Sox2 in neutrophils left unchallenged (Mock) or challenged with 100-nucleotide matched or unmatched *L. monocytogenes* DNA. (c) GST-based precipitation assay of GST alone or GST-tagged full-length Sox2 or Sox2 truncation mutants (above lanes) incubated with histidine-tagged Sox2; below, 10% of the input analyzed without precipitation (loading control). (d) Gel filtration of recombinant full-length Sox2 or Sox2(ΔGBH). (e) Immunoblot analysis of Sox2 in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>-</sup>* and *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils transfected with empty vector or with vector for the expression of full-length Sox2 or Sox2(ΔGBH). (f,g) *In vitro* dimerization assay of GFP-TAK1 and mCherry-TAK1 extracted from HEK293T cells and incubated in the presence (+) or absence (–) of full-length Sox2 (f,g) or Sox2(ΔGBH) (g) and/or *L. monocytogenes* genomic DNA, assessed by confocal microscopy (f) and quantification of those results (g). Scale bars (f), 10 μm.

(h) *In vitro* kinase assay of the IKK complex and IκBα incubated for 1 h with various combinations (above lanes) of *L. monocytogenes* genomic DNA, full-length Sox2, Sox2(ΔGBH), Sox2(ΔSRD), full-length TAB2, TAB2(Δ546–619), full-length TAK1 and/or TAK1 lacking kinase activity (TAK1(K63W)), assessing phosphorylated (p-) and total IκBα (top); below (input), analysis of Sox2, TAB2 and TAK1. (i,j) Immunoblot analysis of phosphorylated and/or total IKK, IκBα, p38, Jnk and full-length Sox2 or Sox2(ΔGBH) (i) and RT-PCR analysis of mRNA encoding proinflammatory cytokines (j) in *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils transfected with empty vector or with vector for the expression of full-length Sox2 or Sox2(ΔGBH), assessed at various times (above lanes, i) or 6 h (j) after mock challenge or challenge with *L. monocytogenes* genomic DNA. (k,l) Immunoblot analysis of phosphorylated and/or total IKK, IκBα, p38, Jnk and human Sox2 (k) and RT-PCR analysis of mRNA encoding proinflammatory cytokines (l) in human neutrophils treated with nontargeting control siRNA (siCtrl) or *SOX2*-specific siRNA (siSOX2), then mock challenged or challenged with the 100-nucleotide matched or unmatched sequence. mRNA results (j,l) are presented relative to those of cells transfected with empty vector or control siRNA, set as 1. \**P* < 0.01 and \*\**P* < 0.001 (Student's *t*-test). Data are representative of three experiments with similar results (mean and s.d. in j,l).



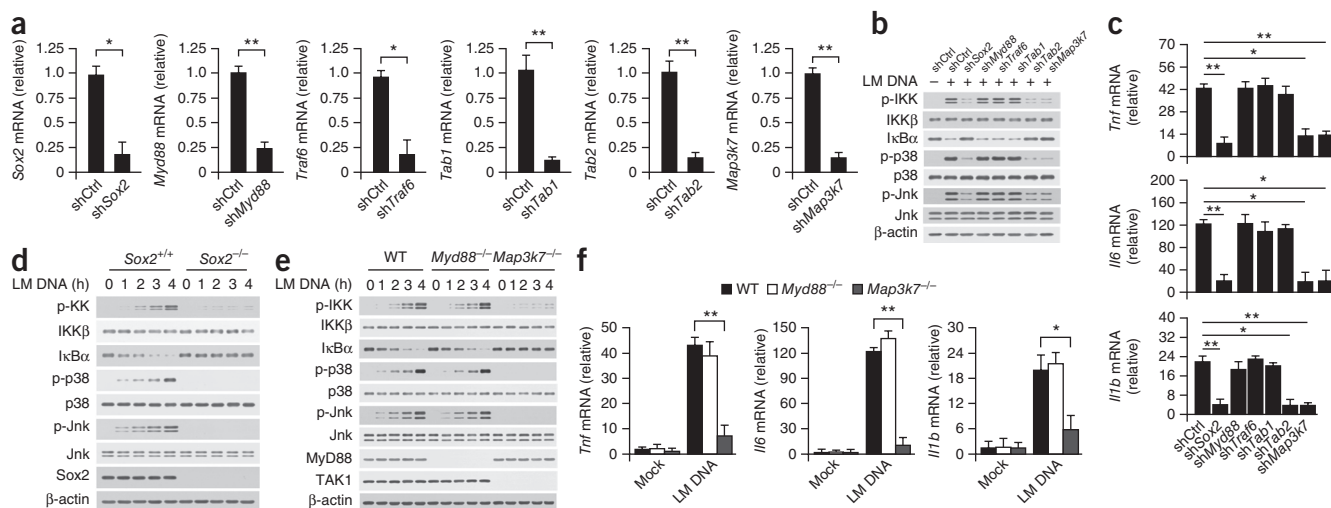
following such infection (Supplementary Fig. 4c,d). Following infection with vaccinia virus, *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>-</sup>* and *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils did not express *Tnf*, *Il6* or *Il1b* (Supplementary Fig. 4e) and did not phosphorylate the kinases Jnk, p38 and IKK (Supplementary Fig. 4f), which suggested a predominant role for macrophages, but not neutrophils, in the response to infection with vaccinia virus.

To map the Sox2 region that interacts with bacterial DNA, we transfected wild-type neutrophils to express Flag-tagged Sox2 mutants lacking various domains (Supplementary Fig. 4g) together with matched 100-nucleotide dsDNA. Deletion of the HMG domain completely abolished the binding of Sox2 to the matched dsDNA, whereas Sox2 with any other mutant domain had a binding affinity similar to that of full-length Sox2 (Fig. 4h). We obtained similar results by an amplified luminescent-proximity homogeneous assay to assess the interaction between two molecules (Fig. 4i). Transfection of *Sox2<sup>fl/fl</sup>* *Lyz2-Cre<sup>+</sup>* neutrophils to express Sox2 lacking the HMG domain failed to induce the expression of *Tnf*, *Il6* or *Il1b* following challenge with bacterial DNA, whereas transfection of these cells to express full-length Sox2 significantly induced the expression

of *Tnf*, *Il6* and *Il1b* (Fig. 4j), which suggested that Sox2 sensed intracellular bacterial DNA in neutrophils through its HMG domain. Overall, these results indicated that Sox2 sensed bacterial DNA in neutrophils in a sequence-specific manner.

### Sox2 associates with TAB2 upon bacterial infection

To define the molecular mechanism by which Sox2 senses DNA in neutrophils, we screened a mouse BM cDNA library using Sox2 as a bait, through a yeast-two hybrid approach. This identified TAB2 as an interacting protein (Fig. 5a). Of 35 positive clones, 23 were identified as TAB2 (Supplementary Fig. 5a). The direct interaction between Sox2 and TAB2 was very weak in *in vitro* precipitation assays (Fig. 5b). However, the addition of *L. monocytogenes* DNA substantially enhanced the association of Sox2 with TAB2 (Fig. 5b). The other candidate proteins identified in the yeast-two hybrid screen showed no interaction with Sox2 in neutrophils transfected to express these proteins (data not shown). We confirmed those observations by immunostaining (Fig. 5c). Notably, when transfected with *L. monocytogenes* DNA, Sox2 associated with TAB2 and TAK1 in



**Figure 7** Sox2 acts upstream of the TAB2-TAK1 axis during bacterial infection. **(a)** RT-PCR analysis of the knockdown efficiency of shRNA targeting Sox2 (shSox2), MyD88 (shMyD88), TRAF6 (shTraf6), TAB1 (shTab1), TAB2 (shTab2) or TAK1 (shMap3k7) in mouse promyelocyte cells; results are presented relative to those of cells transfected with nontargeting control shRNA (shCtrl), set as 1. **(b,c)** Immunoblot analysis of phosphorylated and total components of the NF-κB and AP-1 signaling pathways **(b)** and RT-PCR analysis of mRNA encoding proinflammatory cytokines **(c)** in cells transfected with shRNA as in **a**, assessed before (–) **(b)** or 6 h after **(b,c)** electroporation of *L. monocytogenes* genomic DNA; mRNA results **(c)** are presented relative to those of mock-treated cells. **(d)** Immunoblot analysis of the NF-κB and AP-1 signaling pathways (as in **b**) in *Sox2<sup>fl/fl</sup>* Lyz2-Cre<sup>+</sup> neutrophils at various times (above lanes) after electroporation of *L. monocytogenes* genomic DNA. **(e)** Immunoblot analysis of the NF-κB and AP-1 signaling pathways (as in **b**) in wild-type (WT), MyD88-deficient (*Myd88<sup>-/-</sup>*) and TAK1-deficient (*Map3k7<sup>-/-</sup>*) neutrophils before (0) or various times (above lanes) after transfection of *L. monocytogenes* genomic DNA. **(f)** RT-PCR analysis of mRNA encoding proinflammatory cytokines neutrophils as in **e** left untransfected (Mock) or 6 h after transfection of *L. monocytogenes* genomic DNA. \**P* < 0.01 and \*\**P* < 0.001 (Student's *t*-test). Data are representative of three experiments with similar results (mean and s.d. in **a,c,f**).

neutrophils in a co-immunoprecipitation assay (Fig. 5d). In addition, following the incubation of the 100-nucleotide matched or unmatched dsDNA from *L. monocytogenes* with lysates of wild-type neutrophils, the matched bacterial dsDNA co-precipitated a complex of Sox2, TAB2 and TAK1, while the unmatched bacterial dsDNA failed to precipitate this complex (Fig. 5e). These data suggested that Sox2 associated with the TAB2-TAK1 complex upon stimulation of neutrophils with bacterial DNA.

We next mapped the domain of Sox2 used for interaction with TAB2. We incubated recombinant Sox2 mutants and TAB2 together for *in vitro* precipitation assays and found that the SRD domain of Sox2 was necessary for the interaction with TAB2 (Fig. 5f). Transfection of *Sox2<sup>fl/fl</sup>* Lyz2-Cre<sup>+</sup> neutrophils to express the Sox2 mutant lacking the SRD domain (Sox2(ΔSRD)) did not induce the expression of mRNA encoding proinflammatory cytokines after transfection of *L. monocytogenes* DNA, whereas transfection of these cells to express full-length Sox2 did (Fig. 5g). We incubated recombinant TAB2 and its truncation mutants (Supplementary Fig. 6a) together with recombinant Sox2, followed by *in vitro* precipitation assays. We observed that TAB2 lacking residues 534–619 (TAB2(Δ534–619)) did not bind Sox2 (Fig. 5h) and did not restore the expression of mRNA encoding proinflammatory cytokines when overexpressed in *Tab2<sup>-/-</sup>* neutrophils transfected with *L. monocytogenes* genomic DNA (Fig. 5i), in contrast to overexpression of full-length TAB2, which did ‘rescue’ these effects (Fig. 5h,i). However, TAB2(Δ534–619) interacted with TAK1 (Supplementary Fig. 6b), which suggested that this particular domain of TAB2 (residues 534–619) was required only for interaction with Sox2.

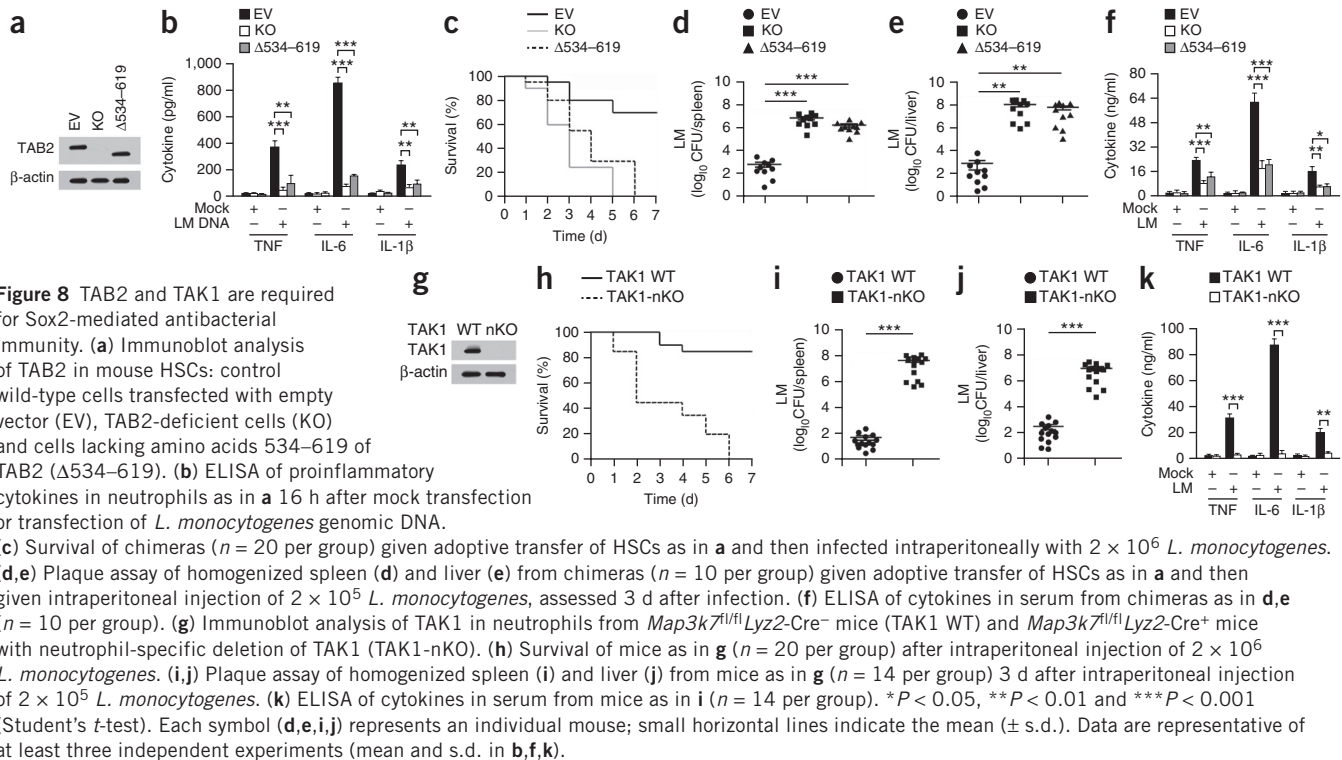
Sox2 is known to control the expression of various sets of genes in the cells in which it functions as a transcription factor. The expression of these target genes was not lost in Sox2-deficient neutrophils (Supplementary Fig. 6c), which suggested that Sox2 did

not function as a transcription factor in the neutrophils. Notably, transfection of *L. monocytogenes* genomic DNA did not cause translocation of Sox2 into the nucleus in neutrophils (Supplementary Fig. 6d). These results suggested that the association of Sox2 with the TAB2-TAK1 complex had a role in the cytokine response to bacterial infection that was independent of its transcriptional activity.

#### Activation of the TAB2-TAK1 complex requires Sox2 dimerization

Non-reducing electrophoresis indicated that Sox2 existed as a monomer and a dimer in neutrophils (Fig. 6a) and that transfection of *L. monocytogenes* genomic DNA augmented formation of the Sox2 dimer (Fig. 6a). In addition, the 100-nucleotide matched DNA triggered more dimerization of Sox2 than that in untransfected neutrophils, while the unmatched DNA did not (Fig. 6b). Analysis of various truncation mutants of Sox2 revealed that the GBH domain was necessary for the dimerization of Sox2 in an *in vitro* precipitation assay (Fig. 6c), and, in contrast to full-length Sox2, a Sox2 mutant lacking the GBH domain (Sox2(ΔGBH)) did not form dimers, as assessed by gel-filtration chromatography (Fig. 6d). When transfected into *Sox2<sup>fl/fl</sup>* Lyz2-Cre<sup>+</sup> neutrophils, Sox2(ΔGBH) did not form dimers, whereas full-length Sox2 did (Fig. 6e). These results indicated that the GBH domain was required for Sox2 dimerization.

To determine whether Sox2 dimerization induces TAK1 activation upon stimulation with DNA, we transfected HEK293T human embryonic kidney cells to express TAK1 tagged with green fluorescent protein (GFP) or the red fluorescent protein mCherry and incubated supernatants of lysates of these cells with full-length Sox2 in the presence of *L. monocytogenes* genomic DNA (Supplementary Fig. 6e). By confocal microscopy, we found that full-length Sox2 induced the association of GFP-tagged TAK1 with mCherry-tagged TAK1 in the presence of bacterial DNA (Fig. 6f). However, even in the presence of genomic DNA, Sox2(ΔGBH) did not induce the co-localization



**Figure 8** TAB2 and TAK1 are required for Sox2-mediated antibacterial immunity. (a) Immunoblot analysis of TAB2 in mouse HSCs: control wild-type cells transfected with empty vector (EV), TAB2-deficient cells (KO) and cells lacking amino acids 534–619 of TAB2 ( $\Delta 534$ –619). (b) ELISA of proinflammatory cytokines in neutrophils as in a 16 h after mock transfection or transfection of *L. monocytogenes* genomic DNA. (c) Survival of chimeras ( $n = 20$  per group) given adoptive transfer of HSCs as in a and then infected intraperitoneally with  $2 \times 10^6$  *L. monocytogenes*. (d,e) Plaque assay of homogenized spleen (d) and liver (e) from chimeras ( $n = 10$  per group) given adoptive transfer of HSCs as in a and then given intraperitoneal injection of  $2 \times 10^5$  *L. monocytogenes*, assessed 3 d after infection. (f) ELISA of cytokines in serum from chimeras as in d,e ( $n = 10$  per group). (g) Immunoblot analysis of TAK1 in neutrophils from *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* mice (TAK1 WT) and *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* mice with neutrophil-specific deletion of TAK1 (TAK1-nKO). (h) Survival of mice as in g ( $n = 20$  per group) after intraperitoneal injection of  $2 \times 10^6$  *L. monocytogenes*. (i,j) Plaque assay of homogenized spleen (i) and liver (j) from mice as in g ( $n = 14$  per group) 3 d after intraperitoneal injection of  $2 \times 10^5$  *L. monocytogenes*. (k) ELISA of cytokines in serum from mice as in i ( $n = 14$  per group). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  (Student's *t*-test). Each symbol (d,e,i,j) represents an individual mouse; small horizontal lines indicate the mean ( $\pm$  s.d.). Data are representative of at least three independent experiments (mean and s.d. in b,f,k).

of GFP-tagged TAK1 and mCherry-tagged TAK1 (Fig. 6g), which suggested that Sox2 dimerization was required for the formation of TAK1 dimers upon bacterial infection.

The NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  is a downstream target of TAK1. I $\kappa$ B $\alpha$  was phosphorylated only in the presence of full-length Sox2, TAB2 and TAK1 as well as *L. monocytogenes* DNA, while Sox2( $\Delta$ GBH), Sox2( $\Delta$ SRD), TAB2( $\Delta 534$ –619) or mutant TAK1 lacking kinase activity failed to phosphorylate I $\kappa$ B $\alpha$  even in the presence of bacterial DNA (Fig. 6h), which indicated that dimerization of Sox2 and formation of the TAB2-TAK1 complex were required for the phosphorylation of I $\kappa$ B $\alpha$ . In contrast to Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup> neutrophils transfected to express Sox2, Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup> neutrophils transfected to express Sox2( $\Delta$ GBH) did not activate the NF- $\kappa$ B and AP-1 signaling pathways following transfection with *L. monocytogenes* genomic DNA (Fig. 6i) and did not induce the expression of mRNA encoding proinflammatory cytokines (Fig. 6j), which indicated that Sox2 dimerization was needed to activate the TAB2-TAK1 complex for neutrophil activation.

We next silenced Sox2 in human peripheral neutrophils through the use of small interfering RNA (siRNA). Transfection of the 100-nucleotide matched DNA, but not of unmatched DNA, induced the activation of NF- $\kappa$ B and AP-1 pathways in human neutrophils-treated with nontargeting control siRNA with a scrambled sequence but not in human neutrophils in which Sox2 expression had been knocked down by Sox2-specific siRNA (Fig. 6k). Consequently, human neutrophils treated with control siRNA expressed mRNA encoding proinflammatory cytokines after transfection of matched DNA but not after transfection of unmatched DNA, while silencing of SOX2 suppressed cytokine expression in these cells (Fig. 6l), which indicated that Sox2-mediated activation of neutrophils was also linked to the clearance of microbes in humans. Together these results indicated that Sox2 triggered activation of the TAB2-TAK1 complex in neutrophils upon stimulation with bacterial DNA.

### Sox2 acts upstream of the TAB2-TAK1 axis in neutrophils

To further determine the physiological role of Sox2 in neutrophils, we silenced genes encoding major molecules upstream of the NF- $\kappa$ B and AP-1 pathways through the use of short hairpin RNA (shRNA) (Fig. 7a). Two shRNAs directed against each individual gene displayed similar knockdown efficiency (data not shown). Knockdown of TAB2 or TAK1 in neutrophils impaired activation of the NF- $\kappa$ B and AP-1 signaling pathways after transfection of *L. monocytogenes* genomic DNA, as determined by immunoblot analysis, while knockdown of the adaptors MyD88 or TRAF6 or TAB1 had no effect (Fig. 7b). Moreover, knockdown of TAB2 or TAK1 abolished the expression of mRNA encoding proinflammatory cytokines following transfection of neutrophils with *L. monocytogenes* DNA, while knockdown of MyD88, TRAF6 or TAB1 had no effect on this expression (Fig. 7c). Consistent with that, activation of the NF- $\kappa$ B and AP-1 pathways following transfection of *L. monocytogenes* genomic DNA was lost in Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup> neutrophils, in contrast to their activation in Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup> neutrophils transfected similarly (Fig. 7d). Activation of NF- $\kappa$ B and AP-1 following transfection of *L. monocytogenes* genomic DNA was normal in *Myd88*<sup>-/-</sup> neutrophils but was lost in TAK1-deficient (*Map3k7*<sup>-/-</sup>) neutrophils (Fig. 7e). Consequently, *Myd88*<sup>-/-</sup> neutrophils had normal expression of mRNA encoding proinflammatory cytokines comparable to that of wild-type neutrophils, while cytokine production in *Map3k7*<sup>-/-</sup> neutrophils was impaired (Fig. 7f). These data suggested that Sox2 initiated the activation of signaling via NF- $\kappa$ B and AP-1 upstream of TAB2 and TAK1 upon bacterial challenge in neutrophils.

### TAB2 or TAK1 deficiency impairs Sox2-mediated immunity

We next investigated the physiological role of the TAB2-TAK1 complex in Sox2-mediated bacterial infection. Using the genome-editing approach noted above (Fig. 3c and Supplementary Fig. 2c,d), we generated mouse HSCs lacking TAB2 due to deletion of exon 3

of *Tab2* or lacking amino acids 534–619 of TAB2 due to deletion of exons 4 and 5 of *Tab2* (Fig. 8a and Supplementary Fig. 7a–d) and adoptively transferred them into lethally irradiated wild-type mice to generate TAB2-deficient BM chimeras and TAB2( $\Delta$ 534–619) BM chimeras, respectively. In contrast to neutrophils from wild-type mice, neutrophils from the TAB2-deficient and TAB2( $\Delta$ 534–619) chimeras did not produce TNF, IL-6 or IL-1 $\beta$  following transfection of *L. monocytogenes* genomic DNA (Fig. 8b). TAB2-deficient and TAB2( $\Delta$ 534–619) chimeras underwent more death following infection with *L. monocytogenes* than did their wild-type counterparts (Fig. 8c), and this corresponded to a higher bacterial load in the spleen and liver of the TAB2-deficient and TAB2( $\Delta$ 534–619) chimeras than in that of the wild-type mice (Fig. 8d,e). The production of proinflammatory cytokines by peripheral neutrophils from TAB2-deficient and TAB2( $\Delta$ 534–619) chimeras *ex vivo* was significantly lower than that of their wild-type counterparts (Fig. 8f).

We also generated mice with neutrophil-specific deletion of TAK1 by crossing *Map3k7<sup>fl/fl</sup>* mice with *Lyz2-Cre* mice (Fig. 8g). More of the resultant *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* mice than their *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>-</sup>* littermates died after challenge with *L. monocytogenes* (Fig. 8h); the *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* mice also had a higher bacterial load in the spleen and liver (Fig. 8i,j) and less secretion of proinflammatory cytokines from peripheral neutrophils (Fig. 8k). Together these data indicated that TAB2 and TAK1 were required for Sox2-mediated antibacterial clearance.

## DISCUSSION

The recognition of cytosolic DNA is important for the detection of pathogens and the induction of host antimicrobial responses. Here we have demonstrated a previously unrecognized function for Sox2 as a cytosolic DNA sensor with a critical role in priming innate immune responses to bacterial infection in neutrophils. Sox2 localized to the cytoplasm of neutrophils and recognized microbial DNA through its HMG domain. Neutrophil-specific Sox2 deficiency exacerbated bacterial infection. Upon challenge with bacterial DNA, Sox2 dimerization was needed to activate the TAB2-TAK1 complex leading to activation of NF- $\kappa$ B and AP-1 signaling cascades in neutrophils. TAB2 or TAK1 deficiency impairs Sox2-mediated antibacterial immunity.

We selected a model of infection with *L. monocytogenes* to investigate the recognition of DNA by Sox2 in neutrophils because *L. monocytogenes* can be ingested by phagocytosis and cleared by neutrophils<sup>30</sup>. Other bacteria, such as *Bartonella*, *Staphylococcus* and *Salmonella*, have Sox2-binding sequences in their genomes that can also be recognized by Sox2 to activate neutrophils for the clearance of microbes. However, whether neutrophils are linked to the clearance of infection with these bacteria remains controversial. Neutrophils have been reported to be dispensable for the clearance of *L. monocytogenes*<sup>31</sup>. In that study, the neutrophil-depleting antibody 1A8 was injected at the time of or after infection with *L. monocytogenes*<sup>31</sup>. Thus, it remains possible that neutrophils were still required very early during infection. When we depleted mice of neutrophils by injecting antibody 1A8 for 1 week before infection with *L. monocytogenes*, we observed that depletion of neutrophils rendered mice more susceptible to infection with *L. monocytogenes* (data not shown), which suggested a role for neutrophils in the clearance of *L. monocytogenes*. This discrepancy might have been caused by differences in the experimental strategies used and needs to be investigated further.

In addition to its canonical localization to the nucleus, Sox2 is also found in the cytoplasm of zygotes before implantation<sup>25,28</sup>. Given that

Sox2 has two nuclear-localization signals, Sox2 may shuttle between these subcellular compartments of ES cells through the use of these two signals. Overexpression of mutant Sox2 lacking these two signals causes the differentiation of ES cells into the trophectoderm lineage<sup>32</sup>, which suggests that shuttling of Sox2 between the nucleus and cytoplasm is critical for maintaining stem cell pluripotency. However, no studies so far have reported the expression pattern and physiological roles of Sox2 in differentiated cells. Here we found expression of Sox2 in the cytoplasm of neutrophils. These data suggested that binding of bacterial DNA to Sox2 may trigger a conformational change in Sox2 that results in dimer formation. On the basis of structural analysis of the caspase-inhibitory BIR1 domain of the apoptosis inhibitor XIAP and the BIR1-TAB1 complex, the BIR1 dimer interacts with TAB1, which then recruits TAK1 into proximity for TAK1 activation<sup>33</sup>. Disruption of BIR1 dimerization abrogates the activation of TAK1, which results in inactivation of the XIAP-mediated NF- $\kappa$ B pathway. Therefore, the butterfly-shaped dimer model formed by the BIR1-TAB1-TAK1 complex might be suitable for the activation of TAK1 induced by Sox2 dimerization in neutrophils.

Various cytosolic DNA sensors have been identified so far<sup>18</sup>. These DNA-binding proteins recognize bacterial or viral DNA to prime the production of inflammatory cytokines and type I interferons<sup>10</sup>. DNA sensors such as IFI16 and DDX41 activate STING for NF- $\kappa$ B activation and interferon transcription<sup>15,16</sup>. DAI interacts with IRF3 and TBK1 after challenge with DNA and initiates innate immune responses to pathogenic infection<sup>13</sup>. AIM2 together with ASC forms inflammasomes after recognizing dsDNA<sup>12</sup>. Notably, IFI16 has also been reported to bind to ASC to promote inflammasome activation under certain circumstances<sup>34</sup>. Most of the known DNA sensors use the STING-TBK1 pathway or inflammasome signaling to trigger innate immune responses. Additionally, Sox2 is uniquely expressed in the cytoplasm of neutrophils and recognizes bacterial DNA in a sequence-specific manner. For a host, sequence-specific recognition for foreign pathogens might necessitate the distinct recognition of self and non-self to adequately eradicate invading threats while avoiding damage to the host cells. Thus, Sox2 functions as a DNA sensor that is specific for the cell type and DNA sequence, which suggests a pivotal role for Sox2 in the distinct recognition of pathogens.

TAK1 and its activator TAB1 participate in the regulation of Wnt and TGF- $\beta$  pathways<sup>35,36</sup>. It has been reported that TAK1 interacts with TAB2 and has an essential role in the NF- $\kappa$ B pathway downstream of IL-1 receptor and TLR pathways<sup>37</sup>. Upon stimulation with IL-1 or lipopolysaccharide, adaptors such as MyD88, TRAF6, TAB2 and TAK1 are recruited to the Toll-IL-1 receptor homology cytoplasmic domain of these receptors<sup>38</sup>. TAB2 binds Lys63-linked ubiquitin chains through its ubiquitin-binding NZF domain to initiate oligomerization or dimerization of TAK1, which leads to the activation of NF- $\kappa$ B and AP-1 signaling pathways. MyD88 acts as a central adaptor shared by almost TLRs. The interaction of TLRs and MyD88 recruits members of the IRAK family of kinases, which results in activation of TRAF6. TRAF6 in turn activates TAK1 in a ubiquitin-dependent fashion<sup>35</sup>. In this study, we found that MyD88-mediated TAK1 activation was not involved in the initiation of the Sox2-mediated NF- $\kappa$ B and AP-1 signaling. In contrast, bacterial DNA triggered Sox2 dimerization via its GBH domain to activate the TAB2-TAK1 complex, which resulted in the initiation of signaling via NF- $\kappa$ B and AP-1 for neutrophil activation. Thus, our findings have revealed a previously unknown activation pathway in neutrophils and may provide potential therapeutic strategies to augment the innate immunity of neutrophils for the treatment of infectious diseases.

## METHODS

Methods and any associated references are available in the [online version of the paper](#).

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

## ACKNOWLEDGMENTS

We thank J. Hao, J. Jia and Y. Teng for technical support. Supported by the National Natural Science Foundation of China (91419308, 81330047 and 31300645), the 973 Program of the MOST of China (2010CB911902 and 2015CB533705) and the Strategic Priority Research Programs of the Chinese Academy of Sciences (XDA01010407).

## AUTHOR CONTRIBUTIONS

P.X. designed and performed experiments, analyzed data and wrote the paper; S.W. performed experiments and analyzed data; B.Y. and Y.D. analyzed data; G.H. and P.Z. performed experiments; and Z.F. initiated the study, and organized, designed and wrote the paper.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

1. Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D. & Zychlinsky, A. Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* **30**, 459–489 (2012).
2. Mantovani, A., Cassatella, M.A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* **11**, 519–531 (2011).
3. Ajibade, A.A. *et al.* TAK1 negatively regulates NF- $\kappa$ B and p38 MAP kinase activation in Gr-1<sup>+</sup>CD11b<sup>+</sup> neutrophils. *Immunity* **36**, 43–54 (2012).
4. Jaeger, B.N. *et al.* Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *J. Exp. Med.* **209**, 565–580 (2012).
5. Nauseef, W.M. & Borregaard, N. Neutrophils at work. *Nat. Immunol.* **15**, 602–611 (2014).
6. Scapini, P. *et al.* The neutrophil as a cellular source of chemokines. *Immunol. Rev.* **177**, 195–203 (2000).
7. Janeway, C.A. Jr. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
8. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
9. Keating, S.E., Baran, M. & Bowie, A.G. Cytosolic DNA sensors regulating type I interferon induction. *Trends Immunol.* **32**, 574–581 (2011).
10. Wu, J. & Chen, Z.J. Innate immune sensing and signaling of cytosolic nucleic acids. *Annu. Rev. Immunol.* **32**, 461–488 (2014).
11. Bauer, M. *et al.* Bacterial CpG-DNA triggers activation and maturation of human CD11c<sup>+</sup>, CD123<sup>+</sup> dendritic cells. *J. Immunol.* **166**, 5000–5007 (2001).
12. Hornung, V. *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**, 514–518 (2009).
13. Takaoka, A. *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448**, 501–505 (2007).
14. Ishii, K.J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451**, 725–729 (2008).
15. Unterholzner, L. *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nat. Immunol.* **11**, 997–1004 (2010).
16. Zhang, Z. *et al.* The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat. Immunol.* **12**, 959–965 (2011).
17. Li, X.D. *et al.* Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* **341**, 1390–1394 (2013).
18. Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z.J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**, 786–791 (2013).
19. Wu, J. *et al.* Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* **339**, 826–830 (2013).
20. Sarkar, A. & Hochedlinger, K. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* **12**, 15–30 (2013).
21. Arnold, K. *et al.* Sox2<sup>+</sup> adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell* **9**, 317–329 (2011).
22. Tsuruzoe, S. *et al.* Inhibition of DNA binding of Sox2 by the SUMO conjugation. *Biochem. Biophys. Res. Commun.* **351**, 920–926 (2006).
23. Lefebvre, V., Dumitriu, B., Penzo-Mendez, A., Han, Y. & Pallavi, B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. *Int. J. Biochem. Cell Biol.* **39**, 2195–2214 (2007).
24. Schepers, G.E., Teasdale, R.D. & Koopman, P. Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. *Dev. Cell* **3**, 167–170 (2002).
25. Avilion, A.A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* **17**, 126–140 (2003).
26. Yamanaka, S. Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell* **10**, 678–684 (2012).
27. Ishii, K.J. *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat. Immunol.* **7**, 40–48 (2006).
28. Wang, S. *et al.* Transient activation of autophagy via Sox2-mediated suppression of mTOR is an important early step in reprogramming to pluripotency. *Cell Stem Cell* **13**, 617–625 (2013).
29. Chen, X. *et al.* Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* **133**, 1106–1117 (2008).
30. Liu, M. *et al.* Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*. *Sci. Rep.* **2**, 786 (2012).
31. Shi, C. *et al.* Ly6G<sup>+</sup> neutrophils are dispensable for defense against systemic *Listeria monocytogenes* infection. *J. Immunol.* **187**, 5293–5298 (2011).
32. Li, J. *et al.* A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *J. Biol. Chem.* **282**, 19481–19492 (2007).
33. Lu, M. *et al.* XIAP induces NF- $\kappa$ B activation via the BIR1/TAB1 interaction and BIR1 dimerization. *Mol. Cell* **26**, 689–702 (2007).
34. Kerur, N. *et al.* IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi sarcoma-associated herpesvirus infection. *Cell Host Microbe* **9**, 363–375 (2011).
35. Chen, Z.J. Ubiquitination in signaling to and activation of IKK. *Immunol. Rev.* **246**, 95–106 (2012).
36. Kanei-Ishii, C. *et al.* Wnt-1 signal induces phosphorylation and degradation of c-Myc protein via TAK1, HIPK2, and NLK. *Genes Dev.* **18**, 816–829 (2004).
37. Chen, Z.J. Ubiquitin signalling in the NF- $\kappa$ B pathway. *Nat. Cell Biol.* **7**, 758–765 (2005).
38. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650 (2011).

## ONLINE METHODS

**Cell culture and transfection.** Mouse neutrophils were sorted from BM or peripheral blood and were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10% heat-inactivated FBS. Mouse promyelocyte cells were cultured in IMDM containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10 ng/ml mouse granulocyte-macrophage colony-stimulating factor and 20% heat-inactivated horse serum. For neutrophil differentiation, mouse promyelocyte cells were incubated for 3 d with 10  $\mu$ M all-trans retinoic acid (ATRA) with replacement of the IMDM described above. Mouse ES cells were cultured in DMEM containing 15% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1 mM nucleotide, 0.1 mM non-essential amino acid and 1,000 units per ml mouse leukemia inhibitory factor. Human ES cell line H1 cells were grown in DMEM (F12) medium supplemented with 20% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 0.1 mM 2-mercaptoethanol and 4 ng/ml human fibroblast growth factor 2.

For neutrophil transfection, neutrophils ( $1 \times 10^7$ ) were resuspended in 100  $\mu$ l Nucleofector Solution buffer (Lonza) containing 5  $\mu$ g DNA or other substrates, followed by transfection with Nucleofector Program Y-001 on an Amaxa nucleofector II device (Lonza). Cells were recovered by incubation for 6 h in IMDM containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10% heat-inactivated FBS, followed by sorting for viable cells by flow cytometry.

**Neutrophil fractionation.** Neutrophils sorted from mouse BM were resuspended for 5 min in cytoplasm-extraction buffer containing 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.075% (vol/vol) NP-40, pH 7.6, and supernatants were collected as cytoplasmic fractions by centrifugation. Pellets were resuspended for 10 min on ice in nuclear extract buffer containing 20 mM Tris-HCl, 400 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM PMSF and 25% (vol/vol) glycerol, pH 8.0. Supernatants were collected by centrifugation.

**Mice.** *Sox2<sup>fl/fl</sup>* mice were from Jackson Laboratory. *Map3k7<sup>fl/fl</sup>*, *Myd88<sup>fl/fl</sup>* and *Lyz2-Cre* mice were from Nanjing Biomedical Research Institute. *Sox2<sup>fl/fl</sup>*, *Map3k7<sup>fl/fl</sup>*, and *Myd88<sup>fl/fl</sup>* mice were bred with *Lyz2-Cre* mice to generate *Sox2<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>*, *Map3k7<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* and *Myd88<sup>fl/fl</sup>Lyz2-Cre<sup>+</sup>* mice. All the mice were on the C57BL/6 background and were maintained under specific pathogen-free conditions with approval by the institutional committee of Institute of Biophysics, Chinese Academy of Sciences.

**Antibodies.** Antibody information is provided in **Supplementary Figure 5b**.

**Flow cytometry.** Mice were killed and cells were obtained from the femur or peripheral blood. Cells were sifted through 50- $\mu$ m cell strainers after removal of red blood cells, followed by staining with fluorescence-conjugated antibodies (**Supplementary Fig. 5b**) and sorting on a BD Influx cell sorter. Data were analyzed with FlowJo 7.6.1 software.

**Immunofluorescence assay.** Immunostaining was performed as described<sup>39</sup>. Neutrophils were made to adhere to coverslips treated with 0.01% poly-L-lysine and were fixed for 10 min with 4% PFA, followed by permeabilization for 20 min at room temperature with 0.5% Triton X-100. After incubation for 30 min with 10% donkey serum for blockade of nonspecific binding, primary antibodies were added for incubation for 2 h at room temperature. Samples were further stained with secondary antibodies (Alexa Fluor 488–donkey anti-mouse IgG (A-21202), Alexa Fluor 488–donkey anti-rabbit IgG (A-21206), Alexa Fluor 594–donkey anti-mouse IgG (A-21203), Alexa Fluor 594–donkey anti-rabbit IgG (A-21207) and Alexa Fluor 405–donkey anti-mouse IgG (A-31553); all from Molecular Probes), followed by visualization by confocal microscopy (Olympus FV1000).

**Immunohistochemistry and Giemsa staining.** Mouse femurs and spleens were frozen and cut into sections, followed by blockade of nonspecific binding with 10% donkey serum, then immunostaining with fluorescence-conjugated antibodies (**Supplementary Fig. 5b**). Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Otherwise, sections were stained

with primary antibodies (**Supplementary Fig. 5b**), followed by staining with enzyme-conjugated secondary antibodies (peroxidase-conjugated rabbit anti-goat IgG (ZB-2306) and peroxidase-conjugated goat anti-rabbit IgG (ZB-2301); both from ZSGB-Bio) and visualization with a Diaminobenzidine (DAB) Histochemistry Kit (Life Technologies) or Vector Blue Substrate Kit (Vector Laboratories). Standard Giemsa staining was performed according to the manufacturer's instructions (Sigma-Aldrich).

**DNA-precipitation assay.** DNA-precipitation assays were performed as described<sup>28</sup>. DNA was synthesized with NH<sub>2</sub>-modification at the 5' end and the two complementary sequences were annealed, followed by coupling to CNBr-activated Sepharose 4B resin (GE Healthcare). For DNA precipitation, recombinant protein or cell lysates were incubated with DNA-linked resin in the presence of Protease Inhibitor Cocktail Set III (Calbiochem), followed by washing with buffer containing 150 mM KCl and immunoblot analysis of precipitates.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation assays were performed as described<sup>40</sup>. Neutrophils transfected with *L. monocytogenes* 100-nucleotide DNA were crosslinked for 10 min at 37 °C by 1% formaldehyde, followed by preclearance with salmon sperm DNA–protein A agarose and then incubation for 6 h with anti-Sox2 (**Supplementary Fig. 5b**). Sox2 was then immunoprecipitated by salmon sperm DNA–protein A agarose. Immunoprecipitates were sequentially washed with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer and TE buffer, followed by elution with elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). The Sox2-DNA complex was reversed by heating for 4 h at 65 °C. DNA was further purified by phenol-chloroform extraction and was precipitated with ethanol, followed by real-time PCR analysis.

**RNA-mediated interference.** RNA-mediated interference was achieved according to the instructions of the pSUPER system (Oligoengine). pSUPER vectors enclosing target sequences (**Supplementary Fig. 5c**) were constructed.

**RT-PCR analysis and primers.** Total RNA was extracted from cells with Trizol reagent, and cDNA was reverse-transcribed with Superscript II (Invitrogen). RT-PCR was performed with a StarScript II Two-step RT-PCR Kit (Genestar) and the appropriate primers (**Supplementary Fig. 5d**).

**Recombinant protein preparation and gel filtration.** Sequence encoding Sox2, TAB2 or TAK1 was cloned into pGEX-6P-1 for expression of GST-tagged protein or into pET-28a for His-tagged protein purification as described<sup>41</sup>. Plasmids were transformed into BL21 (DE3) *Escherichia coli* competent cells and cultured (until they reached an absorbance of 0.6 at 600 nm), followed by induction for 20–36 h at 16 °C with 0.2 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Cells were homogenized and further purified by Ni-NTA resin columns or GST-sepharose columns (GE Healthcare). GST-tagged proteins were cleaved by PreScission Protease (GE Healthcare) when needed. Recombinant Sox2 was loaded onto a Superdex 200 10/300 column and was eluted with elution buffer (50 mM Tris and 150 mM NaCl, pH7.4). Markers containing thyroglobulin (669 kD), ferritin (440 kD), BSA (67 kD),  $\beta$ -latoglobulin (35 kD), RNase A (13.7 kD), aprotinin (6.5 kD) and vitamin B12 (1.3 kD) were used for calibration of the column, and blue dextran 2000 (about 2,000 kilodaltons) was used for determination of the void volume.

**ELISA-based cytokine analysis.** Supernatants from cultured cells or serum were collected at the appropriate times. Cytokines were analyzed by ELISA kits according to the manufacturer's instructions (R&D Systems).

**AlphaScreen.** Purified Sox2 variants and biotinylated DNA were analyzed with an AlphaScreen by incubation at various concentrations according to the manufacturer's instructions (PerkinElmer).

**Yeast-two hybrid screening.** Yeast two-hybrid screening was performed as described<sup>42</sup>. Sequence encoding Sox2 (amino acids 114–319) was cloned into pGBKT7 vector (BD-Sox2). The truncated form of Sox2 (amino acids 114–319) lacked its two nuclear-localization signals. Thus, the Sox2 protein

used for this screen was located only in the cytoplasm. Yeast AH109 cells were transfected with BD-Sox2 and plasmids containing a mouse BM cDNA library (Clontech/Takara) and then plated on synthetic defined medium. Selected clones were isolated and sequenced. The X- $\alpha$ -gal assay was carried out according to the manufacturer's instructions (Clontech/Takara).

**In vitro kinase activity assay.** TAK1 activity was assessed as described<sup>43</sup>. Recombinant Sox2, TAB2 and TAK1 variants were purified from *E. coli*. The IKK complex was purified from lysates of HEK293T cells. <sup>35</sup>S-labeled I $\kappa$ B $\alpha$  was translated *in vitro*. The necessary components were mixed in reaction buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 2 mM ATP, pH7.5), followed by incubation for 1 h at 37 °C, then proteins were separated by SDS-PAGE and visualized by radiography.

**Deletion of *Tab2* and *Tmem173* by technology based on clustered regularly interspaced short palindromic repeats (CRISPR) and Cas9.** Genomic engineering of *Tab2* was achieved with the CRISPR-Cas9 system as described<sup>44</sup> with the following single-guide (sg) RNA sequences: sgRNA upstream of *Tab2* exon 3, 5'-TTGTTAGTTGTGTATTCTG-3'; sgRNA downstream of *Tab2* exon 3, 5'-GGGTGGATAGTCTCCATGC-3'; sgRNA upstream of *Tab2* exon 4, 5'-TCTGCTGCAGAGTTCGCTGC-3'; and sgRNA downstream of *Tab2* exon 5, 5'-TG TCTCGGAGTATTTAGCTT-3'. Wild-type mouse HSCs isolated from BM were infected with lentivirus to introduce the GFP-containing CRISPR-Cas9 vectors. After culture for 3 d *in vitro*, GFP<sup>hi</sup> cells were sorted by flow

cytometry. Cells were analyzed by PCR. PCR products were subcloned and genotyped by DNA sequencing. Genotyped cells were transplanted into recipient mice for further experiments. For deletion of *Tmem173*, exon 3 of *Tmem173* was flanked by sgRNA (5'-ACACCTCAAGGAGACGCCCT-3' and 5'-TGCTGGGTATCAAGTCATCG-3'). Since the CRISPR-Cas9 genotyping data provided only a 'snapshot' of the genotype of the apparently polyclonal cell population, the possibility that not all alleles had been targeted by this approach could not be excluded.

**Statistical analysis.** Student's *t*-test was used for statistical analysis with Microsoft Excel.

39. Xia, P. *et al.* RNF2 is recruited by WASH to ubiquitinate AMBRA1 leading to downregulation of autophagy. *Cell Res.* **24**, 943–958 (2014).
40. Xia, P. *et al.* WASH is required for the differentiation commitment of hematopoietic stem cells in a c-Myc-dependent manner. *J. Exp. Med.* **211**, 2119–2134 (2014).
41. Wang, L. *et al.* Identification of SERPINB1 as a physiological inhibitor of human granzyme H. *J. Immunol.* **190**, 1319–1330 (2013).
42. Xia, P. *et al.* WASH inhibits autophagy through suppression of Beclin 1 ubiquitination. *EMBO J.* **32**, 2685–2696 (2013).
43. Wang, C. *et al.* TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346–351 (2001).
44. Ran, F.A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).